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Amino Acid Substitutions in NS5A Region of GB Virus C and Response to Interferon Therapy

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GB virus C (GBV-C) is related to hepatitis C virus (HCV) and has a similar genomic structure. Some predictors for the efficacy of interferon (IFN) therapy on HCV have been reported: genotype, viral load, IFN dose, and the amino acid substitutions in the NS5A region, designated as the interferon sensitivity determining region (ISDR). To evaluate the correlation between the amino acid substitutions in the GBV-C NS5A region and the response to IFN therapy, single-strand conformation polymorphism (SSCP) analysis was performed in the 12 concomitantly GBV-C–and HCV–infected patients who received IFN therapy at three time points: before, endpoint, and after the IFN therapy. The region in the GBV-C NS5A studied includes the amino acids that exhibit some homology to the ISDR and the various substitutions. By SSCP analysis, amplicons were separated into 1–4 bands, which indicated the existence of heterogeneity in each host. However, the deduced amino acid sequences in these bands exhibited no characteristic differences among these strains irrespective of response to IFN therapy. Of the 32 strains separated by SSCP, 7 strains were responders, and 25 were nonresponders. The mean amino acid substitution, compared with the consensus sequence of nonresponders, was 1.00 ± 0.93 among responders, and 1.40 ± 0.85 among nonresponders (P = NS). No correlation between the amino acid sequence in the GBV-C NS5A region and response to IFN therapy was found, indicating that the GBV-C NS5A region dose not act as the ISDR. ***J. Med. Virol. 57:376–382, 1999.***

KEY WORDS: GBV-C; HGV; single-strand conformation polymorphism (SSCP); ISDR; interferon

INTRODUCTION

Although the etiology of most non-A, non-B hepatitis cases has been explained since the identification of hepatitis C virus (HCV), some cases of hepatitis associated with unknown agents remain. GB virus C (GBV-C) was first detected in patient with non–A-to-E chronic hepatitis and is considered to be the sixth hepatotropic agent reported [Simons et al. 1995; Leary et al., 1996; Linnen et al., 1996]. This virus is related to HCV on the basis of phylogenetic analysis and has a similar genomic structure [Leary et al., 1996; Ohba et al., 1996]. Although interferon-alpha (IFN-α) or -beta (IFN-β) has been used to treat chronic HCV infection, a large number of patients in which IFN therapy is ineffective exist [Davis et al., 1989; Lau et al., 1993; Orito et al., 1994]. Some predictors for the efficacy of IFN that have been reported are the viral genotype, the viral load, and the IFN dose [Shiratori et al., 1997]. A region, designated as the interferon sensitivity determining region (ISDR), has also been identified in the NS5A region of HCV genotype 1b. A correlation between the amino acid sequence of this region and response to IFN therapy has been reported from Japan [Enomoto et al., 1995, 1996; Chayama et al., 1997; Kurosaki et al., 1997], although some inconsistencies certainly exist in the data from the other countries [Hofgärtner et al., 1997; Squardrito et al., 1997; Zeuzem et al., 1997]. The HCV genotype 1b strains with at least four amino acid substitutions in the ISDR were sensitive when compared to HCV-J, whereas the strains identical to HCV-J were resistant to IFN therapy. The correlation between the amino acid substitutions in the NS5A region and the response to IFN therapy has not been investigated, although the sensitivity of GBV-C to

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IFN therapy has already been reported [Tanaka et al., 1996; Lau et al., 1997; Nagayama et al., 1997; Orito et al., 1997; Saiz et al., 1997]. The region in the NS5A region of GBV-C homologous to the ISDR of HCV has also been reported [Takahashi et al., 1997].

The aims of this study were to evaluate the fluctuation in the response of GBV-C strains to IFN therapy using single-strand conformation polymorphism (SSCP) analysis in patients with concomitant GBV-C and HCV infection receiving IFN therapy, and to investigate the correlation between the amino acid sequences of the NS5A region of GBV-C and response to IFN therapy.

The nucleotide sequences in this study will appear in the DDBJ/EMBL/GenBank with the following accession numbers: AB008051 to AB008082 (GBV-C) and AB008083 to AB008094 (HCV).

MATERIALS AND METHODS

Patients

Twelve patients, including 10 patients reported previously [Orito et al., 1997] and 2 new patients, with chronic hepatitis who were positive for both GBV-C RNA and HCV RNA before the treatment, were studied. Eleven patients received IFN-α, with six million units (MU) per day for 2 weeks followed by 6 MU thrice weekly for 22 weeks intramuscularly and 1 patient (patient 3) received IFN-β with 6 MU per day for 6 weeks intravenously. All patients were positive for anti-HCV (EIA-2, Ortho, Raritan, NJ) and negative for both HBsAg (AUSRIA II, Abbott, North Chicago, IL) and antibody to human immunodeficiency virus (Abbott) before IFN therapy. The serum alanine aminotransferase (ALT) levels were measured for all patients (normal range <25 IU/L). Serum samples were obtained serially at three time points, before the commencement of IFN therapy, at the endpoint of IFN therapy, and 6 months after the conclusion of IFN therapy, and were stored at −40°C until use.

Assessment of Response to IFN Therapy

In this study, complete responders (CR) were defined as patients who tested negative for virus RNA, GBV-C, or HCV, at the endpoint of IFN therapy and 6 months after the cessation of IFN therapy. Nonresponders (NR) were patients who showed no virological response to IFN therapy or patients who exhibited a transient response and virological relapse.

RNA Extraction and cDNA Synthesis

Viral RNA was extracted from 100 μL of serum using the SepaGene-RVR kit (Sanko, Tokyo), precipitated with isopropanol, and washed with ethanol. Complementary DNA (cDNA) was synthesized by incubating the RNA samples at 37°C for 1 hr using Moloney murine leukemia virus reverse transcriptase (MMLV-RT; GIBCO-BRL, Gaithersburg, MD) with random primers.

Detection of GBV-C and HCV RNA

GBV-C RNA was detected using the reverse transcription–polymerase chain reaction (RT-PCR) with primers derived from the 5′ untranslated region (5′ UTR) [Kato T et al., 1997] and HCV RNA was also detected using RT-PCR with primers derived from the 5′ UTR [Okamoto et al., 1990].

Genotyping and Quantification of GBV-C and HCV

The genotypes of GBV-C were divided into three groups in this study: the GB type, the HG type, and the Asia type, which corresponded to genotypes 1, 2, and 3, respectively, according to Muerhoff et al. [1996], using restriction fragment length polymorphism (RFLP) analysis [Mukaide et al., 1997]. The HCV genotype was determined by a mixed primer PCR as described previously [Ohno et al., 1997]. Briefly, the first PCR was performed with common primer to all genotypes, which located in core region. The second round of PCR was performed with mixed primer, containing common sense primer and genotype specific antisense primers. Genotypes of HCV were decided by bands of the genotype specific size. Quantification of GBV-C was performed using competitive RT-PCR [Orito et al., 1997]. Competitive RT-PCR was performed with the same primers to the second round of PCR for detection of GBV-C/HGV. Extracted GBV-C/HGV RNA was reverse-transcribed with known amounts of deleted GBV-C/HGV RNA, which had 20 base deletions in the sequence of amplified region. The cDNA derived from a sample and deleted GBV-C/HGV RNA were coamplified with the same condition of the second round of PCR for GBV-C/HGV detection. Amounts of GBV-C/HGV RNA were determined by comparing signal intensities of PCR products derived from targeted RNA with those from known amounts of deleted RNA. The detection range of this competitive RT-PCR assay was 10^3 to 10^8 copies/mL. Quantification of HCV was by a branched DNA amplification assay (bDNA; Quantiplex HCV RNA version 1, Chiron, Emeryville, CA).

Determination of the ISDR Sequences of HCV

The ISDR sequence of HCV genotype 1b was amplified using nested RT-PCR as reported by Enomoto et al. [1995] and sequenced directly with M13 primers. The ISDR sequence of HCV genotype 2 was amplified using seminested RT-PCR. The first round of PCR was performed with primers c2-5Af3, 5′-TGGGTGGACGGAGTGCAGATCC-3′, and c2-5Ar3, 5′-GGTTGATAATCTGGCCTCTTCCA-3′, for 30 cycles, each consisting of denaturation for 1 min at 94°C, annealing for 45 sec at 55°C, and extension for 1 min at 72°C. The second round of PCR was performed with primers c2-5Af3 and c2-5Ar2,5′-CATATATTCCGATGGTATCGAAGG-3′, for 35 cycles under the same conditions as in the first round of PCR. The amplicons were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide, and observed under ultraviolet light. The

TABLE I. Backgrounds and Clinical Characteristics of the Patients

				GBV-C/HGV			HCV		
Patient	Age	Sex	ALT (IU/L)	Genotype	RNA level[a] (log copies/mL)	IFN response[b]	Genotype	RNA level[c] (Meq/mL)	IFN response[b]
1	28	M	86	Asia	7.5	NR	2a+2b	1.3	NR
2	54	F	236	Asia	7.0	NR	1b	3.8	NR
3	59	M	23	Asia	8.0	NR	1b	30.0	NR
4	26	M	141	Asia	7.0	NR	1b	30.9	NR
5	27	F	124	Asia	7.0	NR	1b	7.1	NR
6	40	M	120	Asia	7.0	NR	1b	6.6	NR
7	58	M	458	Asia	5.0	NR	2b	<0.5	NR
8	36	M	106	Asia	7.5	NR	1b	<0.5	CR
9	60	M	61	Asia	7.5	NR	1b	0.6	CR
10	61	M	98	Asia	6.5	NR	1b	<0.5	CR
11	60	F	65	Asia	3.0	CR	1b	1.2	NR
12	63	F	207	Asia	4.0	CR	1b	1.7	NR

[a]GBV-C/HGV RNA level was measured by competitive RT-PCR.
[b]CR, complete responder; NR, nonresponder.
[c]HCV RNA level was measured by branched DNA assay.

nucleotide sequences of the amplicons were determined directly by the dideoxy chain termination method. The resulting amino acid sequences were compared with the ISDR sequence identified in HCV-J (accession number D90208), and those of HCV genotype 1b were divided into three groups, wild type, intermediate type, and mutant type, as reported by Enomoto et al. [1995].

PCR of GBV-C RNA for SSCP Analysis

GBV-C RNA was detected using nested PCR with specific primers based on the sequence of the NS5A region of GBV-C. This region contains the stretch of 12 amino acid residues that exhibit some homology to the ISDR of HCV in their first half, and the portion that exhibits the most varied amino acid substitutions among the NS5A region of GBV-C and involves 12 amino acid insertion in some strains in their latter half, as suggested by Takahashi et al. [1997]. The first round of PCR was performed with primers g5Af1, 5′-CTATCGGCTGCTGTAGCTGAGCCC-3′, and g5Ar2, 5′-AAGGCTTTTAGTACGGAAAGAGC-3′, for 30 cycles, each consisting of denaturation for 1 min at 94°C, annealing for 45 sec at 60°C, and extension for 1 min at 72°C. The second round of PCR was performed with primers g5Af3, 5′-ACCATTGACGGGGAGCGC-TACACC-3′, and g5Ar1, 5′-TACGGAAAGAGCCACGT-TGAAGAC-3′, for 35 cycles under the same conditions as for the first round of PCR. The expected amplicon size was 402 bp (corresponding to nucleotides 6698 to 7100 of HGV PNF2161) [Linnen et al., 1996].

Single-stranded cDNAs of GBV-C and HCV were amplified by asymmetric PCR using 1% of the first PCR product as a template. Asymmetric PCR was performed using the same primers, the same concentration of sense primer, a 10% dilution of antisense primer, and under the same conditions as for the second round of PCR [Enomoto et al., 1994].

SSCP Analysis

Five μL of single-stranded cDNA was mixed with 10 μL of loading buffer containing 95% formamide, 20-mM EDTA, and 0.1% xylene cyanol. The mixture was loaded on a nondenaturing polyacrylamide gel (MDE gel, FMC Bioproducts, Rockland, ME) and electrophoresis was carried out at 20°C, 100 V, for 6 hr. The gel was stained with ethidium bromide and the bands were observed under ultraviolet light. Separated bands were excised from the gel, and cDNA was recovered by incubation at 60°C overnight. The recovered cDNA was amplified by the asymmetric PCR again and subjected to SSCP analysis to confirm that each band was amplified differently and was homogeneous. Subsequently, using PCR with the same primers and conditions as for the second round of PCR, cDNA was amplified, and the nucleotide sequences of the amplicons were determined using the dideoxy chain termination method. For statistical analysis, the Mann-Whitney U-test was used.

RESULTS

The backgrounds of patients, ALT levels, viral genotypes, viral RNA levels, and responses to IFN therapy are shown in Table I. All patients were infected by the same type (Asia type) of GBV-C. Following IFN therapy, 10 patients were nonresponders with respect to GBV-C, and the remaining 2 patients (patients 11 and 12) were responders with respect to GBV-C. Ten patients were infected with HCV genotype 1b, one patient (patient 1) with HCV genotype 2a + 2b, and one other (patient 7) with HCV genotype 2b. Following IFN therapy, nine patients were nonresponders with respect to HCV, and the remaining three patients (patients 8, 9, and 10) were responders with respect to HCV. None of the patients were responders with respect to both viruses, HCV and GBV-C.

The results of SSCP analysis and the deduced amino acid sequences of each band are shown in Figure 1. In three patients (patients 1–3), GBV-C RNA was detectable at the endpoint of IFN therapy. In seven patients (patients 4–10), GBV-C RNA was undetectable at the endpoint of IFN therapy but became detectable 6 months after the conclusion of IFN therapy. In two

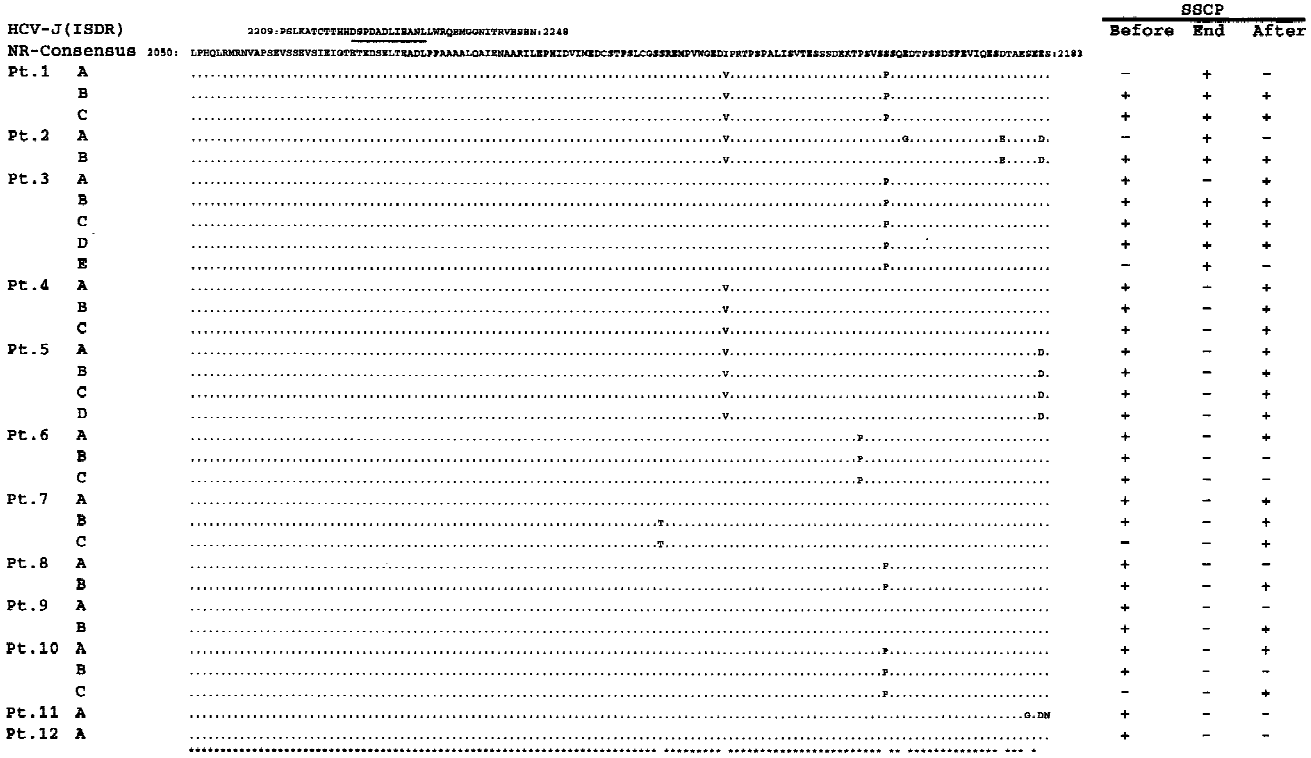

Fig. 1. Alignment of amino acid sequences of GBV-C in NS5A. These sequences were deduced from the nucleotide sequences obtained from the SSCP bands. HCV-J (accession number D90208) is indicated to show the homology between the NS5A region of GBV-C and the ISDR of HCV. A horizontal bar indicates the stretch of 12 amino acid residues that exhibit some homology to the ISDR. NR consensus represents consensus sequence of nonresponder strains and indicated as the reference sequence of nonresponder strains. These nucleotide sequences are numbered according to the sequence of HGV/PNF (accession number U44402). Identical nucleotides are indicated by dots. On the right side of the sequences results of SSCP are shown.

patients (patients 11 and 12), GBV-C RNA remained undetectable at 6 months after the conclusion of IFN therapy. The single-stranded cDNA of GBV-C could be separated into 1–4 bands by gel electrophoresis at each time point in each patient, and thus revealed the existence of heterogeneity in this region. Some bands disappeared after the cessation of IFN therapy (patients 8A, 9A, 10B, 11A, and 12A). Some other bands appeared at the endpoint of IFN therapy (patients 1A and 3E) or at 6 months after the cessation of IFN therapy (patients 6B, 6C, and 10C). The strains of GBV-C represented by these bands had 1–4 different nucleotide sequences among each strain (data not shown). However, the deduced amino acid sequences exhibited no characteristic differences among these strains, irrespective of response to IFN therapy. Moreover, the region flanking the stretch of 12 amino acid residues exhibiting some homology to the ISDR of HCV exhibited the same amino acid sequence among all strains in all the patients. No strains having a 12 amino acid insertion were observed [Takahashi et al., 1997]. Of the 32 strains separated by SSCP analysis, 7 strains were responders and 25 strains were nonresponders. The mean amino acid substitutions, compared with the consensus sequence of nonresponders strains (NR consensus), was 1.00 ± 0.93 among responders and 1.40 ± 0.85 among nonresponders. This difference was not statistically significant (Table II).

TABLE II. Mean Amino Acid Substitutions Compared With NR Consensus

IFN response	Number	Amino acid substitutions[a]
Responder	7	1.00 ± 0.93[b]
Nonresponder	25	1.40 ± 0.85[b]
Total	32	1.31 ± 0.88

[a]The number of amino acid substitutions are compared with the consensus sequence of nonresponders and expressed as mean ± SD.
[b]P = not significant.

The deduced amino acid sequences in the ISDR of HCV in each patient are shown in Figure 2. Of the seven nonresponders with HCV genotype 1b, the wild type was observed in six patients (patients 2–6 and 11) and the intermediate type in one patient (patient 12). Of the three complete responders with HCV genotype 1b, the intermediate type was observed in two patients (patients 9 and 10) and the mutant type in one patient (patient 11). Two patients with HCV genotype 2 (patients 1 and 7) did not responds to IFN therapy, and the amino acid sequences in the ISDR had at least 12 substitutions, and a deletion of 4 amino acids, similar to the prototypes of HCV genotype 2 (HCV-J6 and HCV-J8).

		IFN Response	Genotype	Type of ISDR
HCV-J (1b)	2209: PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN:2248			
HCV-J6(2a)	...R.....GKAY.V.MVD...F-----...DV..I...S			
HCV-J8(2b)	.....KTAY.C.MVD...F-----...DV..I..DS			
Pt.1	.....KMAY.C.MVD....----....V..I..DS	NR	2b	
Pt.2	.....	NR	1b	Wild
Pt.3	.....	NR	1b	Wild
Pt.4	.....	NR	1b	Wild
Pt.5	.....	NR	1b	Wild
Pt.6	.....	NR	1b	Wild
Pt.7	.....KMAY.C.MVD...F-----...DV..I..DS	NR	2b	
Pt.8	......RA.C.N.H.T..T.S.....D	CR	1b	Mutant
Pt.9	.....V.....	CR	1b	Intermediate
Pt.10	V....A...R.....	CR	1b	Intermediate
Pt.11	.....	NR	1b	Wild
Pt.12	.....E.....	NR	1b	Intermediate

Fig. 2. Alignment of amino acid sequences in ISDR of HCV. Identical nucleotides and gaps are indicated as dots and dashes, respectively. HCV-J (accession number D90208), HCV-J6 (accession number D00944), and HCV-J8 (accession number D10988) are shown as the prototype of HCV genotype 1b, genotype 2a, and genotype 2b, respectively. The type divided by comparison with HCV-J corresponded to the criteria of Enomoto et al. [1996].

DISCUSSION

In an earlier study, it was suggested that most GBV-C shows transient sensitivity to IFN therapy, that the pretreatment level of GBV-C RNA predicts the efficacy of IFN therapy [Orito et al., 1997], and that the sensitivity to IFN differs among GBV-C strains in the same host [Kato et al., 1998]. Recently, the domain that contains the stretch of 12 amino acid residues which have some homology to the ISDR of HCV was reported by Takahashi et al. [1997]. However, the relationship between the amino acid sequences of this region and the response to IFN therapy has not been elucidated. In this study, the correlation between the amino acid sequences in the NS5A region of GBV-C, which includes the domain that exhibits homology to the ISDR of HCV and the portion that exhibits the most varied amino acid substitutions and involves 12 amino acid insertion in some strains, and the response to IFN therapy was investigated. As a result, some important findings were suggested: GBV-C exhibits heterogeneity in the NS5A region; the sensitivity to IFN is different among GBV-C strains in the same host, as observed in the case of HCV; and the amino acid sequence in the NS5A region is not correlated with response to IFN therapy.

SSCP analysis is used to detect nucleotide substitutions in single-strand cDNA, by mobility shifts depending on their sequence-specific conformations, and can evaluate the fluctuation of the quasispecies population independently in the host. This method was used for the basic identification of the ISDR of HCV [Enomoto et al., 1994, 1995]. Using this method, a different response of each strain to IFN therapy in the same host was revealed. The deduced amino acid sequences of these strains exhibited no characteristic difference between sensitive and nonsensitive strains in the same patient, or between the strains isolated from complete responder and from nonresponders. The number of bands separated by SSCP tends to correlate with the response to IFN therapy, although the number of patients studied was small. The number of bands may reflect the GBV-C RNA level. The genotype of GBV-C in all the patients in this study was the same i.e., the Asia type, the major genotype in Japan. Studies on larger numbers of patients and on people from other countries are necessary.

Regarding HCV, six of the seven nonresponders with HCV genotype 1b had the wild type and the remaining one had the intermediate type. One of the three complete responders with HCV genotype 1b had the wild type and the others had the intermediate type. Thus, the response to IFN therapy was related to the number of amino acid substitutions in the ISDR [Enomoto et al., 1996]. These concurrent data regarding HCV suggest that the conflicting data regarding GBV-C are probably not due to host factors such as disorders of the immune system. GBV-C is known to exhibit a lower degree of variation than HCV in the genomic region [Pickering et al., 1997; Viazov et al., 1997]. The NS5A region of GBV-C, unlike HCV, may not have different functions among the strains. Recently, some findings on the function of the NS5A region of HCV were reported. The protein encoded by the NS5A region (NS5A protein) of HCV can bind and inhibit the function of PKR, a cellular protein induced by IFN [Gale et al., 1997]. If the function of the NS5A protein of HCV is related to the response to IFN therapy, it will be necessary to establish a functional assay of the NS5A protein of GBV-C and to investigate whether the NS5A protein of GBV-C has the same function as that of HCV. Another report reveals that the NS5A protein of HCV includes two acidic domains and one proline-rich region, and functions as a transcriptional activator [Kato N et al., 1997; Tanimoto et al., 1997]. The NS5A protein of GBV-C similarly has two acidic domains in the middle of the NS5A region investigated in this

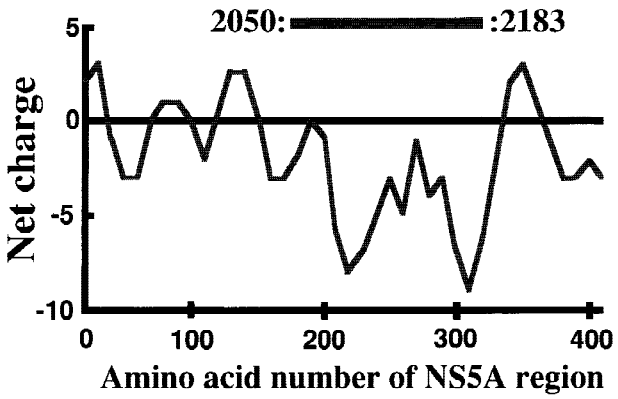

Fig. 3. Charge distribution in NS5A region of GBV-C/prt. Values of −1 for D or E and +1 for K or R were used. The average of net charges over successive 30 amino acids, measured at 10 amino acid intervals, is shown as described [Ma and Ptashne, 1987; Tanimoto et al. 1997]. A horizontal bar indicates the region performed SSCP analysis in this study.

study (Fig. 3). These domains are conserved among all the strains identified in this study and among previously reported strains [Simons et al., 1995; Leary et al., 1996; Linnen et al., 1996; Takahashi et al., 1997]. However, the proline-rich region observed in HCV is absent in GBV-C. This difference may explain the functional difference between the NS5A proteins of HCV and GBV-C, and the reasons for the conflicting data regarding GBV-C in this study.

The two patients with HCV genotype 2, having at least 12 substitutions and a deletion of 4 amino acids compared with HCV-J, did not respond to IFN therapy, although HCV genotype 2 is regarded as sensitive to IFN therapy [Orito et al., 1994]. The relationship between sequence variation in the NS5A region of HCV genotype 2 and the response to IFN therapy is under investigation. At this point, more examples and further studies are required.

In summary, no correlation between the amino acid sequences in the NS5A region of GBV-C and a response to IFN therapy was revealed. The NS5A region of GBV-C, which includes the stretch of amino acid residues exhibiting some homology to the ISDR of HCV, does not act as the ISDR, although the amino acid sequences in the NS5A region of HCV acted as the ISDR in the same patients.

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